EXHIBIT (8 pages)

Assembling the molecular cast

Inspired by the many examples of molecular recognition occurring in nature, Evgeny Vulfson, Cameron Alexander and Mike Whitcombe are finding new ways of mimicking such events in the laboratory

The availability of materials that can bind selectively to a target molecule is the key to unlocking a host of new technologies. Faced with the problem of preparing a material with designed recognition elements, chemists have a number of options open to them.

The first is to turn to biology, harnessing the immune system to raise an antibody to the compound of interest. This is now a routine exercise and there are numerous companies that can do it for a moderate fee, though antibodies are still too expensive for most applications in the chemical industry.

A second possibility is to design and build a receptor molecule from scratch; chemists have synthesised a wide range of structures, including cages, bowls, clefts and crowns, from readily available precursors. The trouble is that the final product is often as expensive as antibodies.

A third option, which may not have been considered until recently, is to prepare an imprinted polymer, capable of combining the advantages of synthetic plastics, such as low cost, durability and robustness, with the recognition properties of natural receptors.

Molecular imprinting exploits the simple, but elegant, principle of using elements of the target molecule to create its own recognition site. This is achieved by forming a highly crosslinked polymeric matrix around a template, which can be the target molecule itself or a close structural analogue. The key to this procedure is to ensure that functional groups of the template molecule fully interact with complementary functional groups of polymer-forming components during polymerisation (*Fig 1*). These interactions are then frozen in by incorporating the whole assembly into the polymer structure; subsequently removing the template reveals the new binding sites.

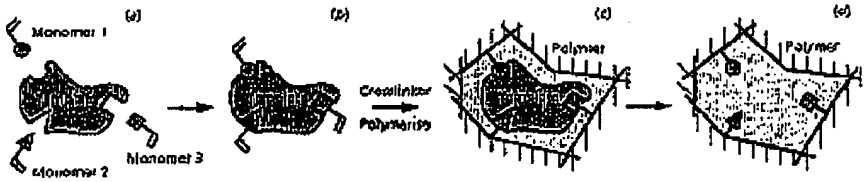


Fig 1. The imprinting process: (a) the template is matched by functional monomers; (b) a templatemonomer complex or covalent adduct forms; (c) the template assembly

copolymerises with a crosslinking monomer; and (d) removing the template reveals the recognition site

Constructing molecular imprints is crucially different from guesthost chemistry. The main difficulty in preparing a synthetic host is often not the design of its recognition elements but the chemical effort required to combine precisely these elements into a single receptor molecule. Imprinting overcomes this problem by holding the recognition elements in place, owing to their interactions with the template, while they are connected to a macromolecular scaffold via growing polymer chains. This allows the pathways between neighbouring groups in the recognition site to be of virtually any length through the crosslinked matrix, precisely matching the template's requirements. An analogy can be made with the structure of antibodies, where amino acid residues at the binding site are brought together by folding the protein chain. Linus Pauling once speculated that antibodies were synthesised to complement the 'template' antigen. This insight proved to be incorrect, but was the first description of molecular imprinting.[3]

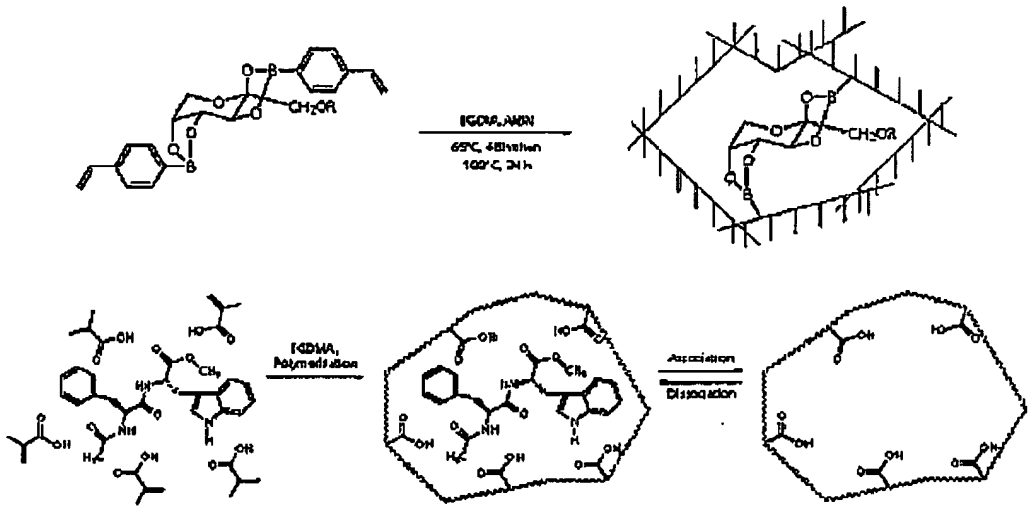
Inevitably, the most critical decisions in synthesising an imprinted polymer are choosing what functional groups are involved in recognition and how to introduce them into the binding site. The latter is usually achieved by either covalently modifying the template molecule with suitable polymerisable species, or by forming a non-covalent complex between the template molecule and the functional monomers *in situ* (*see Box*).

Covalent and non-covalent methods

Covalent imprinting is being pioneered by Günter Wulff and coworkers at the University of Bonn and, more recently, in Dusseldorf, Germany. The aim of this method is to produce an 'exact fit' recognition site, in which the same chemical bonds in the initial templatemonomer complex reform during any subsequent binding of the imprinted polymer cast.

For this to be true, it must be possible to form a labile, reversible bond between the template and the polymer. Thus, 4-vinylphenylboronic acid, which forms cyclic boronate esters with diols, can be used to imprint monosaccharide derivatives,[17] (*Scheme a*). Removing the template from the resulting polymer requires only mild hydrolysis with, for example, aqueous methanol, leaving a polymer cavity containing boronic acid residues in the exact spatial arrangement necessary to rebind the template. Besides boronate esters, the formation of ketals and Schiff's bases also involves reversible covalent bond formation, giving rise to a useful, although somewhat restricted, range of functional groups (diols, aldehydes, ketones and primary amines) for covalent imprinting.

Non-covalent imprinting, pioneered by Klaus Mosbach's group at the University of Lund in Sweden, has been applied to a much wider range of template molecules including amino acid derivatives,[18] peptides,[17,18] b-blockers,[5] diazepam[7] and theophylline.[7] It relies on self-assembling functional monomers around the template in the polymerisation mixture (Scheme b) and, because no covalent bonds form between the template and polymer, template removal involves simply washing the polymer repeatedly with a suitable solvent. Although a range of functional monomers are used, methacrylic acid and vinylpyridine are among the most common. Again, the rebinding of non-covalently imprinted polymers is an 'exact-fit' process since the interactions between the polymer and its target ligand (usually a drug or other metabolite) will be the same as those holding together the templatemonomer complexes. As a result, factors that tend to increase the stability of the initial complex will improve imprinting efficiency, while those that destabilise it will produce an inferior polymer. Binding to these polymers can be very rapid, because covalent bond formation is not involved in recognition.

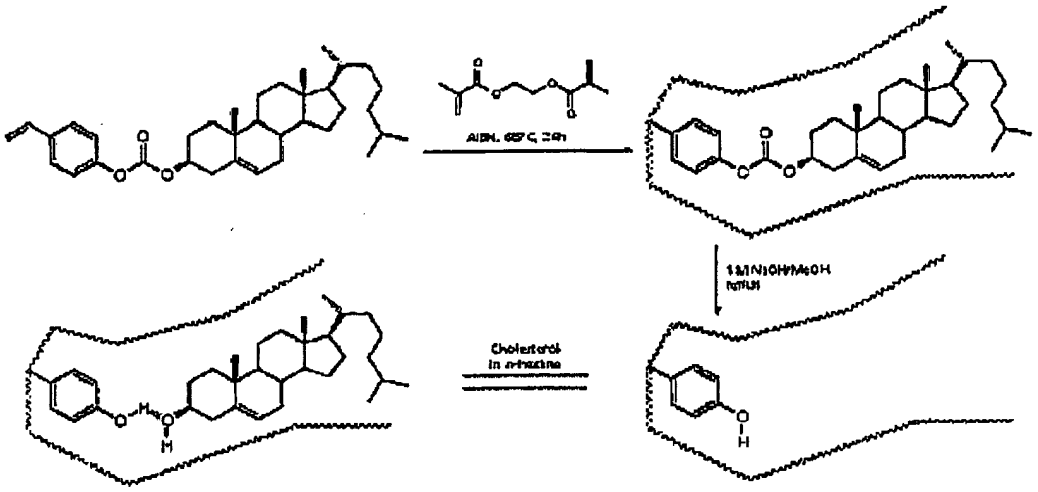


Both strategies have advantages and disadvantages. The attraction of non-covalent imprinting is the simplicity and versatility of the procedure, which can address elements of the template structure that are not amenable to covalent imprinting, by using a range of chemical interactions (ionic, dipolar and H-bonding). The major drawback is the unavoidable diversity of binding sites obtained due to the multitude of complexes that form between monomers and template during the initial stages of polymerisation.

Taking the example in *Box* scheme b, we would expect all the species: peptide, peptide plus one, two, three, four or five methacrylic acid molecules - in all their permutations - to be present in equilibrium in the reaction mixture. Moreover, the need to maximise non-covalent interactions responsible for complex formation during polymerisation limits the choice of solvents and reaction conditions.

In principle, it should be possible to use a mixture of monomers, each designed to interact with a particular functional group of the template molecule, but this is rarely done in practice to avoid preferential association of complementary or multi-functional monomers with each other rather than with the template. This problem should not arise in covalent imprinting, because the arrangement of functional groups in the recognition site should be independent of the other monomers and reaction conditions. However, even the most successful strategies, involving boronate esters, are incompatible with some solvents, *eg* alcohols.

At the Institute of Food Research, we have been investigating the possibility of developing an alternative imprinting strategy, combining the advantages of covalent and non-covalent methods. Our approach is to use a covalently modified template for the initial stage of polymerisation, and to rely on non-covalent interactions for the recognition event. In practice, this has led us to develop a new 'sacrificial spacer' methodology, shown in *Scheme 1*. In this example, we prepared a cholesterol-specific polymer by imprinting with cholesteryl (4-vinyl)phenyl carbonate as the template monomer.[4] Hydrolytic cleavage of the carbonate ester, with the loss of 'sacrificial' CO_2, led to a recognition site bearing a phenolic residue.



Scheme 1. Imprinting cholesterol by the sacrificial spacer method

The resulting polymers show preferential binding of cholesterol over epi-cholesterol, cholest-5-ene-3-one and cholesteryl acetate. Remarkably, they also bind cholesterol with a single dissociation constant, thus displaying characteristics similar to true biological receptors or synthetic hosts. We are currently developing this method with the aim of introducing other functionalities, for example amino, amido, hydroxyl, carboxyl and thiol groups, in the polymer's recognition site. If successful, this will provide us with the same kit of functional groups that Nature uses to construct the binding sites of enzymes and antibodies.

Designing a recognition site and selecting an appropriate imprinting strategy is just the first step in making an imprint. The next is to prepare the polymer. Free radical vinyl polymerisation is the most common method of forming imprinted polymers. The main characteristic of polymers prepared in this way is their high degree of crosslinking due to a large proportion (typically between 60 and 95 per cent) of a di- or tri-functional monomer in the polymerisation mixture. Common crosslinkers include divinylbenzene, ethyleneglycol dimethacrylate (EGDMA) and, more recently, trimethylolpropane trimethacrylate, which is superior to EGDMA in some applications. This high level of crosslinking serves a dual purpose: it provides an element of rigidity in the recognition sites by forming the supporting matrix and ensures that the matrix is sufficiently porous to allow the ligand to diffuse in and out of the recognition site. The polymerisation solvent also has a multiple role: it solubilises the monomer before polymerisation, stabilises any non-covalent complexes and acts as a 'porogen', helping to control the porosity of the resulting polymer.

Imprinted polymers are generally prepared as a monolith, under conditions resembling bulk polymerisation, rather than by emulsion or suspension polymerisation techniques favoured by industry. This is because neither non-covalent imprinting, involving water-soluble monomers such as methacrylic acid, nor covalent imprinting with boronates (which are readily hydrolysable), can easily be carried out in aqueous reaction media. Not only should water be avoided, but many other solvents could also interfere with the mechanism of imprinting, limiting the choice of porogen.

Our sacrificial spacer method suffers from none of these disadvantages owing to the stability of the templatemonomer. This has allowed us to prepare imprinted polymers as porous beads, using suspension polymerisation techniques (*Fig 2*), and we are currently investigating a two-stage method for preparing similar particles with a uniform size distribution. Making imprinted beads involves adjusting the reaction conditions, which is far from trivial, but the resulting polymers are essentially the same as those prepared in bulk, in a much more convenient form.

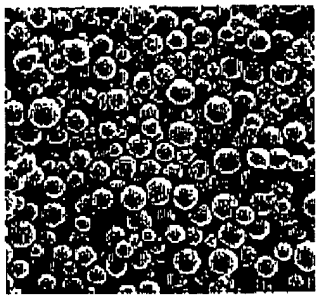
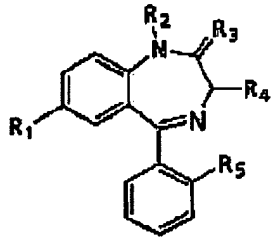


Fig 2. SEM of cholesterol-imprinted polymer beads prepared by suspension polymerisation

One of the major driving forces for developing imprinting techniques is the realisation that polymers, imprinted with a single enantiomer, should be able to resolve racemic mixtures. Initial work has concentrated on model systems, including racemates of monosaccharides, amino acid derivatives and even peptides. However, Mosbach and coworkers recently reported separating b-blockers[5] and non-steroidal anti-inflammatory drugs,[6] which are more industrially significant. Both separations involved non-covalent imprinting using EDGMA with methacrylic acid as the functional monomer. This is followed by analytical scale HPLC, using the imprinted polymer as the stationary phase, to resolve the enantiomers. Predictably, the enantiomer used for imprinting has a longer retention time and elutes as a broader peak compared with that of its mirror image.

Another spectacular application of imprinted polymers, also developed by Mosbach's group, is in highly sensitive radioassays for detecting very small quantities of drugs, pesticides or other metabolites.[7] These assays are based on the competition for receptor binding between the metabolite and its radioactively labelled form bound to the imprinted polymer. The higher the concentration of free metabolite in a sample, the more labelled template is displaced from the polymer, and hence the more radioactivity released into solution. Researchers have detected as little as 6 nM of the drug (S)-propanolol by using this method.[8] Equally remarkable, in a study of the competition for binding diazepam drugs in *Table 1*, their imprinted polymer counterparts demonstrate almost the same cross-reactivity as the corresponding antibodies.

Table 1. Cross-reactivities of benzodiazepines determined by competitive binding of ^{3}H-diazepam to diazepam-imprinted polymer and to diazepam antibodies [7]



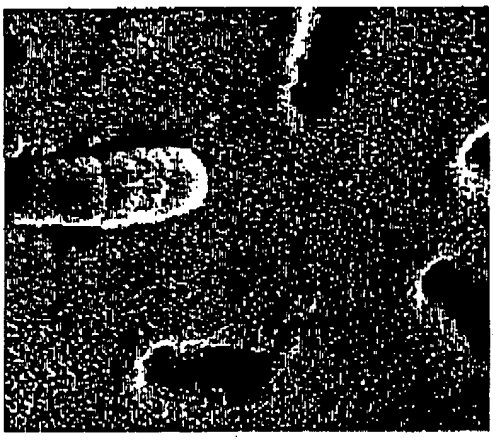
Competitive Ligand	R_1	R_2	R_3	R_4	R_5	*Cross-reaction (%) polymer*	*Cross-reaction (%) antibody*

Diazepam	Cl	Me	O	H	H	100	100
Alprazolam	Cl			H	H	40	44
Desmethyldiazepam	Cl	H	O	H	H	27	32
Clonazepam	NO_2	H	O	H	H	9	5
Lorazepam	Cl	H	O	OH	Cl	4	1

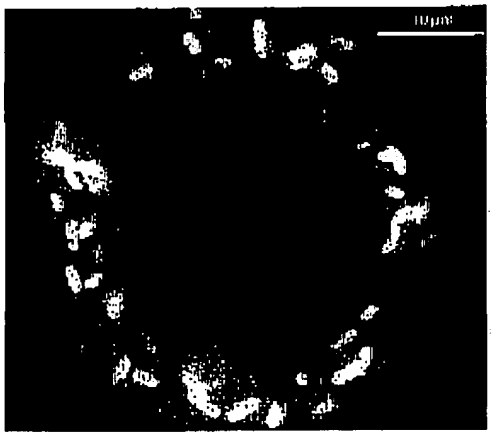
Because imprinted polymers generally work better in organic rather than aqueous media, they could replace antibodies in some clinical and/or environmental analyses involving poorly water-soluble substances. They are also likely to become useful for detecting low levels of environmental pollutants, such as herbicides and pesticides. Several groups, in the US,[9] Japan[10] and Sweden,[11] have recently reported using imprinted polymers to detect low levels of the herbicide atrazine, and the closely related herbicide prometryn.[12] These radioassays are not as convenient and portable as enzyme linked immunosorbent assays (Elisa), but they nevertheless show great promise.

As well as imprinting small organic molecules, we can extend the technique to encompass more complex templates such as proteins or even whole cells. At the Institute of Food Research we have used microorganisms as the template to yield polymer surfaces with functional sites exactly complementary in size and shape to those of bacterial cells.[13]

The key to this multi-stage process, best described as bacteria-mediated lithography, is to use the bacteria as 'temporary protecting groups' while chemically modifying the surrounding polymer surface. Removing the template microorganisms leaves 'bug-sized' imprints, visible by scanning electron microscopy (*Fig 3a*). The process offers a generic method for creating template-defined, functionally anisotropic, patches on the surface of polymeric beads (*Fig 3b*), which we can further modify with antibodies or other biomolecules to enhance their binding characteristics. The resulting materials may find wide-ranging applications in medicine and environmental/food analysis for efficiently separating and recovering cells and viruses.



(a)



(b)

Fig 3. (a) SEM of Listeria monocytogenes and imprints on a polymeric surface; and (b) Scanning confocal laser micrograph of lithographic 'prints', showing the difference in chemistry between the imprinted sites and the surface of the beads

Perhaps even more attractive is the idea of using imprinted polymers as selective adsorbents in the chemical industry. Plastic components with antibody-like properties, which could withstand aggressive solvents, extreme temperatures and pressures, would be useful for separating individual compounds from complex mixtures, or for removing undesirable components or contaminants present at low concentration during waste treatment. They should be easy to regenerate and, if necessary, sterilise (particularly important for biotechnological applications), without loss of activity.

Much has been made of imprinted polymers being an inexpensive alternative to antibodies. It is hardly surprising therefore that the tenets of catalytic antibody research have also been borrowed by workers on imprinted polymers. The principle involved is that raising an antibody to a transition state analogue for a reaction of interest should produce a protein capable of stabilising the actual transition state, hence accelerating the reaction.

Imprinted polymer catalysts, prepared in a similar way, are yet to match enzymes or even catalytic antibodies in terms of rate enhancements.[14,15] This is partly due to the involvement of just a single functional monomer in the catalytic sites of polymers prepared so far, compared with the highly cooperative interactions of numerous amino acid residues in the active sites of biological catalysts. New and better strategies for introducing multiple functional groups, capable of cooperative action, is imperative for further progress. However, the true potential of 'printzymes' lies not in their ability to compete with proteins but to complement them, as robust catalysts made for particular reactions for which no enzymes can be found. As an example of this new kind of catalyst, researchers have recently used a rhodium-containing polyurethane, imprinted with a chiral ligand, to catalyse the reduction of ketones to alcohols, yielding an enantiomer ratio consistent with a 'memory' for the chiral template.[16]

More work is still required to develop a better understanding of structure-function relationships in imprinted polymers, and hence a more rational approach to polymer design. To those of us involved in this research, it offers the satisfaction of tackling challenging scientific problems while, at the same time, preparing smart polymers for a variety of practical uses. The potential of 'plastic antibodies' has already been amply demonstrated and the first industrial processes using imprinted polymers are just beginning to emerge.

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Further reading

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